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(54) Title: IMPROVED VACCINE

SEQUENCE DESCRIPTION TB-2

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GGATCCCTAC GTCACGCTTA ACAATAAAC AACAAAAATG AGAAAAACAA TCAACAACT 60
AGAGGTTTCAG ATTTAAGATC TTGTTTTTTT CATTAGCGT CTGATGAGTC CGTGAGGAGC 120
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ATGTTGTTTC GCGGCTGTA TGAGTCCCTG AGGACGAAC GCTTAAGGAT CC 1432

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SEQUENCE DESCRIPTION TB-1

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ATCCAGTGAG ATGACAGACT CAAGCTAGAA TTACAGGCTA CTCTCTGAA CACTGGGTA 360
TCATCGGACC AACTCCAAGA TAGATAAATA TCCCTCGAA TAAGTTGTT TCTGAAGTAA 420
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AGCTCAAGAT CATCCACCAT CTTGTGACT AGGAAGGACT CTACATCAT GTCAATCAGA 720
GTATGATCA TCTCGTTGA AGGATTGAT ATGATCTCTT CAGGAATCC CCTTACCAGG 780
TCTTGAATG TTAAGGAACG AGCTCTCTGC ATCTCACTT TGGGCTGCT GCAATTGAAC 840
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AAACCTCTG ACCGAAGGAA CGCTCTCGAC AGATGTTGAA TAGCTTTATA GTCCGGGTTT 1020
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CGCTTAAGGA TCC 1393

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(57) Abstract

A virus like particle (VLP) containing an RNA genome including a 3' domain and a filler domain surrounded by a sheath of rabies M protein or similar protein from a rhabdovirus or paramyxovirus and rabies M₁ protein or similar protein from a rhabdovirus or paramyxovirus to form a ribonucleo protein complex. The VLP also includes a lipid envelope of rabies G protein or similar protein from a rhabdovirus or paramyxovirus and an internal matrix of rabies M₂ protein or similar protein from a rhabdovirus or paramyxovirus. There is also included a process for constructing the VLP and also a vaccine for treatment of rhabdovirus and paramyxovirus infection which utilises the aforementioned VLPs in association with an adjuvant. There is also included a DNA construct for use in the abovementioned process.

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**IMPROVED VACCINE
TECHNICAL FIELD**

THIS INVENTION relates to an improved vaccine, and in particular to vaccines for treatment of infections caused by rhabdoviruses which include rabies virus, bovine ephemeral fever virus (ie BEFV) and vesicular stomatitis virus (ie VSV), and paramyxoviruses which include mumps virus and measles virus of humans, respiratory syncytial viruses of humans and cattle, rinderpest virus of cattle, canine distemper virus of dogs, Newcastle disease virus of chickens and various parainfluenza viruses.

BACKGROUND ART

Rabies is a disease of the central nervous system of major importance to human and veterinary medicine. The disease causes a fatal encephalomyelitis for which there is no treatment once the disease symptoms have appeared. Vaccination either before or after virus contamination is then the only way to combat the infection. Various vaccines are licensed for human, veterinary and domestic animal use and all are prepared from killed virus. To date, subunit vaccines have not been used commercially.

The etiological agent of rabies is a rhabdovirus genus *lyssavirus*. Rabies virions contain a ribonucleoprotein (RNP) consisting of a negative stranded (-) RNA molecule approximately 12,000 nucleotides long surrounded by a protective sheath of N protein. The RNP is associated with two other proteins (L and M₁) and together this structure forms the transcription complex. The transcription complex is surrounded by a lipid bilayer membrane associated with 2 proteins which comprise a transmembrane glycoprotein (G) and an internal matrix protein (M₂). The G protein forms the spikes visible on the surface of virions. Other rhabdoviruses and paramyxoviruses have a similar structure, although the number and function of the proteins can be different.

The foregoing synopsis on rabies will be found in Prehaud et al (1989) *Virology* 173: 390-399 and Prehaud et al (1990) *Virology* 178: 486-497. A summary of the structure of some other rhabdoviruses and paramyxoviruses will be found in *The Rhabdoviruses* (1987) Ed. RR Wagner Plenum Press, New York; Emerson (1985) in *Virology* Ed. BN Fields pp 1157-1178; Wunner et al (1985) In *Immunochemistry of Viruses* Vol 1 Ed. MHV Van Regenmortel and AR Neurath Elsevier Press, Amsterdam pp 367-388; and Orvell and Norrby (1985) In *Immunochemistry of Viruses* Ed. MHV Van Regenmortel and AR Neurath pp 241-264.

It has been made clear in Prehaud et al (1989) that the problems associated with vaccination against rabies are far from being resolved. While vaccines have been improved in regard to both vaccine quality and availability, and concomitant advances have been made in rabies diagnostics, epidemiology and surveillance, the disease continues to be a threat to human and animal populations.

Types of vaccine that have been developed or considered as candidates include the following:

(i) Inactivated rabies virus preparations.

These vaccines are currently in use for humans and domestic animals. They are safe to employ except when the virus is incompletely inactivated. The major problem with these vaccines is the cost of production.

(ii) Live attenuated vaccines.

These vaccines have been used for the oral vaccination of wildlife animals. For such purposes it is a prerequisite that candidate vaccines are avirulent for both the target species and for the non-target animals that may be occasionally infected. The candidate live vaccine has also to be immunogenic and genetically stable. In recent years, new attenuated virus strains have been prepared having several mutations that affect virulence.

(iii) Recombinant poxviruses. These include

recombinant fowlpox virus and recombinant vaccinia virus. Both have been shown to be effective vaccines. However, it is not known whether release into the environment of live recombinant poxviruses poses other risks for wildlife.

(iv) Subunit proteins or structures.

Vaccines derived from subunit proteins or structures of the virus have been proposed. However, the cost of these vaccines is high. Several types of subunit vaccine have been proposed and these include G-M₂ complexes, as described in Benmansour (1985) Ann. Inst. Pasteur Virol. 136E:167-173, and immunostimulating complexes (ISCOMs) described in Morein et al. (1984) Nature (Lond.) 308:457-460. Liposome vaccines have been described in Perrin et al (1985) Dev. Stand. Biol. 160: 483-491, and vaccines based on ribonucleoproteins have been described in Dietzschold et al. (1983) Proc. Natl. Acad. Sci USA 80:70-74 and Fu et al (1991) Proc. Natl. Acad. Sci. USA 88: 2001-2005. The G protein expressed in insect cells has been tested as a vaccine in mice [Prehaud et al (1989) above].

In regard to VSV, it is known that this virus contains 5 structural proteins and that each of these proteins plays a role in the replication, assembly and budding of VSV. These include the nucleocapsid protein (N), the phosphoprotein (NS), and the large polymerase protein (L) which with the viral RNA genome form a RNP transcription complex similar to that described above for rabies virus. There is also included a glycoprotein (G) which forms the spikes on the viral envelope and which interacts with receptors on susceptible cells. There is also a matrix protein (M) which appears to be similar to the M₂ protein of rabies virus and is thought to assemble at the inner surface of the cellular plasma membrane to allow association with the G protein and the RNP complex during particle morphogenesis. A summary of the structure and morphogenesis of VSV will be found in

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Pattniak and Wertz (1991) Proc. Natl. Acad. Sci. USA 88: 1379-1383 and in *The Rhabdoviruses*(1987) Ed. RR Wagner Plenum Press, New York.

5 VSV infects horses, cattle, swine as well as
humans and previous vaccines have been derived from
inactivated or killed virus or attenuated virus as
described above in relation to conventional rabies
vaccines. The same problems in relation to possible
conversion from avirulent to virulent forms is also
10 relevant to VSV vaccines. Subunit vaccines have not been
extensively investigated although immunity may be
obtained through a subunit vaccine based on the G protein
[Cox et al (1977) *Infection and Immunity* 16: 754-759; Le
Francoi (1984) *J. Virol.* 51:208]. A vaccine based on the
15 combination of the N and G proteins has also been
reported in Dietzschold et al (1983) referred to above.

In relation to BEFV, this virus causes an acute
infection of cattle and water buffalo. Vaccines that
have been produced so far include live attenuated viruses
20 or inactivated whole virus. Such vaccines have so far
not proved to be commercially successful and also suffer
from the risks of incomplete inactivation or reversion to
virulence as describe above. BEFV as described in
Australian Patent Specification 61356/90 and in Walker et
25 al. (1991) *J Gen Virol.* 72:67-74 comprises an envelope
glycoprotein (G), a nucleoprotein (N), a matrix protein
(M₂), a polymerase-associated protein (M₁) and a large
polymerase protein (L). Australian Patent Specification
61356/90 refers to the use of a subunit vaccine based on
30 the G protein. However, such a vaccine has not been
produced commercially.

Paramyxoviruses and rhabdoviruses are both
classified as virus Families (Paramyxoviridae and
Rhabdoviridae respectively) in the Order Mononegavirales.
35 These Families of viruses share broadly similar
structures, genome organisation and strategies of gene
expression and replication. Viruses of both Families

have a single-stranded (-) sense RNA genome which incorporates 3' and 5' terminal domains which are involved in nucleation of particle assembly, and at least 5 viral genes including those encoding a nucleoprotein, matrix protein, polymerase protein, glycoprotein and an RNA-dependent RNA polymerase. These corresponding proteins have the same general role in viral replication in both Families. The structure of paramyxovirus particles involves an RNP complex which associates with a matrix protein and buds through the cellular plasma membranes to incorporate glycoproteins in a similar process to that described above for rabies virus. Paramyxoviruses include important human and veterinary pathogens including measles virus, mumps virus, parainfluenza viruses, respiratory syncytial viruses, Newcastle disease virus of chickens, rinderpest virus and canine distemper virus. Various inactivated, live attenuated, recombinant and subunit protein vaccines against paramyxoviruses have been described and these have similar properties to those described above for rhabdovirus vaccines. A discussion of the similarities of rhabdoviruses and paramyxoviruses is in Pringle (1991) Arch. Virol. 117:137-140 and a summary of the structure of paramyxoviruses and paramyxovirus vaccines is in Ray and Compans (1990) in *Immunochemistry of Viruses* Vol II Ed. MHV Van Regenmortel and AR Neurath Elsevier Press, Amsterdam pp 217-236.

It has recently been demonstrated that synthetic virus-like particles (VLPs) can be constructed by expressing viral structural genes in cultured eukaryote cells. The procedure has been used to construct synthetic VLPs of poliovirus. Urakawa et al. (1989) J. Gen. Virol 70: 1453-1463 have reported the insertion of the complete polycistronic mRNA of poliovirus in the baculovirus polyhedrin gene. Insect cells infected with the recombinant baculovirus have synthesised and processed the poliovirus polyprotein and

generated large quantities of empty VLPs. These synthetic capsids contained no RNA and were not infectious but were otherwise similar to native poliovirus. Similar methods have been used to construct (core-like particles) CLPs and VLPs of several other viruses including bluetongue virus [French and Roy (1990) J. Virol. 64:1530-1536; French et al (1990) J. Virol. 64:5695-5700], hepatitis B virus [Takehara et al (1988) J. Gen. Virol. 69:2763-2777] and bovine immunodeficiency virus [Rasmussen et al (1990) Virology 178: 435-451]. To date, the particles formed by this method have been generated by protein-protein interactions and have contained no viral nucleic acid (RNA or DNA).

Related technology has been used to recover infectious virus from cDNA clones representing the entire genome of some viruses. By this method infectious cDNA has been cloned into plasmid vectors containing promoters suitable for expression in eukaryote cells. Transfection of eukaryote cells with such vectors has resulted in the production of infectious virus. This general approach has been used in relation to a number of viruses of humans, plants and animals including poliovirus [Racaniello and Baltimore (1981) Science 214:916-919], Sindbus virus [Rice et al (1987) J. Virol. 61:3809], swine vesicular disease virus [Inoue et al (1990) J. Gen. Virol 71: 1835-1838] and brome mosaic virus [Ahlquist et al. (1984) Proc. Natl. Acad. Sci. USA 81:7066-7070]. The method applies only to RNA viruses with a positive sense (+) genome which can function directly as a messenger RNA. This method does not apply to rhabdoviruses and paramyxoviruses which have a negative (-) sense RNA genome.

DISCLOSURE OF THE INVENTION

The objection of this invention to provide an effective and completely non-infectious vaccine for rhabdoviruses and paramyxoviruses contain a minus sense (-) RNA genome and so infectious or defective particles

cannot be generated from cDNA clones representing the entire genome or a genome fragment alone. These viruses also require assembly elements located on the RNA for particle formation and so VLPs cannot be formed by protein-protein interactions alone.

The process of the invention therefore includes the following steps:

(i) constructing a DNA molecule corresponding to a modified genome or genome fragment of a rhabdovirus or paramyxovirus. The DNA molecule may comprise a 3' domain and at least one ribozyme domain and optionally a 5' domain and may incorporate cohesive ends; and

(ii) inserting the DNA obtained in step (i) into the cloning site of a eukaryote expression vector and transfecting a eukaryote cell with the vector containing the genome construct and simultaneously transfecting the same eukaryote cell with vectors containing cloned genes of rhabdovirus or paramyxovirus structural proteins including those with similar functions to the G protein, N protein, M₁ protein and M₂ protein of rabies virus; and

(iii) obtaining from the cell transfected in step (ii), virus-like particles (VLPs) consisting of an RNA genome transcribed from DNA molecule constructed in step (i), surrounded by a sheath of N protein and M₁ protein to form a ribonucleoprotein complex and a lipid envelope including the G protein and an internal matrix comprising the M₂ protein; and

(iv) including the VLPs obtained in step (iii) in a vaccine.

A general structure for a range of suitable DNA constructs for use in the invention is illustrated in Figure 1 and the general process of the production of the desired VLPs is summarised schematically in Figure 2.

In relation to step (i) of the process of the invention, the 5' and 3' domains may be derived from the

sequences of the 5' and 3' non-coding regions of the genome of a rhabdovirus or paramyxovirus although the 5' domain may be deleted if necessary. The ribozyme domain or domains may be constructed from any of the known ribozyme structures, some of which are described by Haseloff and Gerlach (1988) Nature 334:585-591. The ribozyme domain(s) will ensure that, in step (ii) of the process, extraneous parts of the RNA genome construct transcribed in eukaryote cells [such as vectors sequences and a poly(A) tail] will be cleaved at the appropriate location in the molecule to allow particle assembly [as described in step (iii)]. The ribozyme domains may be cleaved from the RNA transcript before assembly into VLPs or included in the transcript provided it does not prevent the assembly process. Each of the ribozyme domains may be located externally of the 3' and 5' domains and intervening nucleotide sequences may be interposed between the domains of the construct. Alternatively, the ribozyme domain(s) may be located internally of the 3' and 5' domains as shown in Figure 1.

The filler domain may constitute any nucleotide sequence that has characteristics which will not prevent the formation of VLPs. Preferably, the filler domain will constitute a fragment derived from a portion of the L protein coding region of a rhabdovirus or paramyxovirus which is adjacent to the 5' terminal non-coding region of the (-) RNA genome. The filler domain will ensure that the genome to be expressed in step (iii) will be sufficient size (greater than approximately 1000 nucleotides) to allow formation of VLPs.

Specific examples of DNA constructs suitable for formation of rabies VLPs are illustrated in Figures 3-8. DNA sequences which are shown in illustrations are presented as single-stranded molecules in the sense in which the construct will be transcribed. Double-stranded DNA molecules that may be required in certain constructs will incorporate a second strand of anti-complementary

sequence.

Figures 3, 4 and 5 illustrate the structural organisation and sequence of a suitable DNA construct (TB-2) which includes two ribozyme domains (R1 and R2). In this example, the 5' and 3' domains are derived from the known nucleotide sequence of the 5' and 3' terminal regions of the genome of rabies virus (PV and CVS strains). The R1 domain is designed to target a site within the (-) RNA transcript of the TB-2 DNA construct. The R1 ribozyme in the transcript will cleave the RNA to ensure that extraneous parts of the transcript are removed so that the 5' terminus of the transcript corresponds to that of the 5' terminus of the rabies virus genome. Similarly, the R2 ribozyme domain is designed to target a site within the (-) RNA transcript of the TB-2 DNA construct. The R2 ribozyme will cleave the RNA to ensure that extraneous parts of the 3' region of the transcript (including the R2 domain) are removed so that the 3' terminus of the transcript approximates that of the 3' terminus of the rabies virus genome. The filler domain in the TB-2 construct is derived from the known nucleotide sequence of a 1135 nucleotide region at the 5' end of the rabies virus (CVS strain) L protein gene.

Figures 6, 7 and 8 illustrate organisation and sequence of a suitable DNA construct (TB-1) which incorporates a single ribozyme domain (R). In this example, the 5' and 3' domains are derived from the known nucleotide sequence of the corresponding 5' and 3' terminal regions of the genome of rabies virus (PV and CVS strains). The R Ribozyme domain is designed to target a site within the (-) RNA transcript of the TB-1 DNA construct. The R ribozyme will cleave the RNA to ensure that extraneous parts of the 3' region of the transcript (including the R domain) are removed so that the 3' terminus of the transcript approximates that of the 3' terminus of the rabies virus genome. The filler

domain in the TB-1 construct is derived from the nucleotide sequence of a 1167 nucleotide region at the 5' end of the rabies virus (CVS strain) L protein gene.

5 In relation to step (ii) of the process of the invention, it will be appreciated by the person skilled in the art that any suitable vector may be used to express the modified genome or genome fragment and viral structural proteins. This may include, for example, eukaryote systems, eg mammalian cells using poxvirus, 10 papillomavirus or retrovirus vectors or in yeast cells. The preferred expression system is, however, the use of a baculovirus vector to infect an insect host cell such as that from *Spodoptera frugiperda*.

In regard to the process of the invention it is 15 known that large quantities of proteins may be produced relatively cheaply and easily by using insect cell culture and baculovirus expression systems. This is described in Cameron et al. (1989) TIBTECH 7:66-70. Baculoviruses are large DNA viruses which infect insects. 20 Late in the infection cycle baculoviruses express several proteins in very large quantities. The genes that express these proteins (eg polyhedrin and p10) are not essential for baculovirus replication in culture and have been used as cloning sites for foreign genes. Such 25 recombinant baculoviruses express foreign eukaryote or viral proteins in high levels and in a form which often closely resembles the native protein. A large number of animals virus proteins have been expressed in the baculovirus system under the control of the p10 or 30 polyhedrin promoters. Examples of the application of the baculovirus system for expression of viral proteins are provided in Emery (1991) Reviews in Medical Virology 1:11-17. Examples of the use of the baculovirus system for expression of rhabdovirus proteins have been provided 35 in Bailey et al (1989) Virology 169: 323-331, Prehaud et al (1989) Virology 173:390-399 and Prehaud et al. (1990) Virology 178:486-497 and for paramyxovirus proteins in

Van Wyke Coelingh et al (1987) Virology 160:465-472 and Vialard et al. (1989) described the expression of the G protein. The rabies G protein gene was cloned and inserted into a baculovirus transfer vector pAcYM1 derived from the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV). The recombinant transfer vector and AcNPV DNA were used to co-transfect *Spodoptera frugiperda* cells and a recombinant baculovirus which expressed high levels of the rabies G protein was recovered from the cells.

Prehaud et al. (1990), referred to above, describes the preparation of a baculovirus expression vector (AcNPV3) derived from *Autographa californica* nuclear polyhedrosis virus and containing the complete coding region of the N protein of rabies virus. The rabies gene was placed under the control of the AcNPV polyhedrin promoter and was expressed in high levels by the derived recombinant baculovirus in *Spodoptera frugiperda* cells. The baculovirus expression system is also reported in, for example, Smith et al. (1985) Proc. Natl. Acad. Sci. USA 82:8404-8408; Miller et al. (1986) Genetic engineering: Principles and Methods 8:277-298; Possee (1986) Virus Res. 5:43-59; Matsuura et al (1987) J. Gen. Virol. 67:1515-1529; Lucknow et al. (1988) Biotechnology 6:47-55; Kang (1988) Adv. Virus Res. 35:177-192; Bishop and Possee (1990) Adv. Gene Technol. 1: 55-72; and Miller et al (1988) Ann. Rev. Microbiol. 42: 177-199.

In relation to the formation of synthetic VLPs, it is known that the natural formation of rhabdovirus and paramyxovirus particles requires viral structural proteins but does not require the complete genomic RNA. For example, during the course of rhabdovirus or paramyxovirus infections of animals or cell cultures defective-interfering (DI) particles are commonly formed. DI particles may contain all viral structural proteins but contain a deleted and hence defective genome. The

defective genome renders the DI particles incapable of replication in the absence of complete, non-defective virus. Aspects relating to the nature of DI particles are reviewed in Huang and Baltimore (1977) Comprehensive Virology 10:73-116; Holland et al (1980) Comprehensive Virology 16:137-192; Perrault (1981) Curr. Top. Microbiol. Immunol. 93:151-207; and Holland (1985) In Virology Ed. BN Fields Raven Press, New York pp 77-99. It is also known in Pattniak and Wertz (1991) Proc. Natl. Acad. Sci. USA 88:1379-1383 that replication of VSV DI particles can occur when cells are infected simultaneously with VSV DI particles and vaccinia virus vectors which expresses all 5 VSV structural proteins from cloned cDNA.

15 In relation to steps (iii) and (iv) of the process of the invention, by using methods described and illustrated for the rhabdovirus TB-2 and TB-1 DNA constructs, it would be possible to produce synthetic rhabdovirus or paramyxovirus defective or infectious virus-like particles (VLPs) in insect cells. VLPs produced by this method would require no helper virus or DI particles and no helper cells. After assembly and release from cells, the VLPs may be utilised as a suitable vaccine in combination with suitable adjuvants as is known in the art. Such adjuvants include Quil A and other saponins, ISCOMs, Freund's incomplete or complete adjuvant, and any other adjuvant as described for example in Vanselow (1987) Vet. Bull 57:881-896.

30 It will also be appreciated that using the VLPs as described above will contain all of the important immunogenic proteins of the native virus presented in a form which closely resembles the native structure. When used in a vaccine for administration to subjects who may suffer from a disease or complaint caused by rhabdoviruses or paramyxoviruses, the VLPs will cause immunity in much the same way as vaccines incorporating inactivated viruses are presently used. However, unlike

vaccines incorporating rhabdovirus or paramyxovirus particles, there is no possibility that infectious virus will be present or that reversion to virulence will occur because the VLPs of the present invention may use only a
5 fragment of the viral genome and no genes encoding complete viral proteins.

It will also be appreciated that the principles and strategies described for construction VLPs based on rabies virus may be applied to any other (-)sense non-
10 segmented RNA virus, particularly rhabdoviruses and paramyxoviruses. Essentially, the required VLP will contain a suitable modified genome or genome fragment containing essential assembly elements including a 3' domain, corresponding to the 3' terminal sequence of the
15 genome of the rhabdovirus or paramyxovirus, at least one ribozyme domain to ensure that the expressed RNA is cleaved to produce the required 3' terminus and a filler domain to ensure that the expressed sub-genomic RNA has sufficient size to nucleate particle formation
20 (approximately 1000 nucleotides). The construct may also incorporate a 5' domain which will perfectly or imperfectly base pair with the 3' domain and a second ribozyme domain to ensure that the expressed RNA is cleaved to produce the required 5' terminus. The
25 transcript of the required DNA construct will then be co-expressed in eukaryote cells with the required structural proteins of the homologous rhabdovirus or paramyxovirus to all VLP formation.

PREFERRED MODE OF CARRYING OUT THE INVENTION

30 The process of the invention is described in the following examples which are illustrative of the invention but in no way limiting on its scope.

ABBREVIATIONS AND DEFINITIONS

| | |
|----------|---|
| 35 ACNPV | Autographa californica nuclear polyhedrosis virus |
| BEFV | Bovine ephemeral fever virus |
| cdNA | Complementary deoxyribonucleic acid |

| | | |
|----|---------|---|
| | CLP | Core-like particles |
| | CVS | Challenge virus standard |
| | DI | Defective-interfering |
| | DNA | Deoxyribonucleic acid |
| 5 | EDTA | Ethylenediamine-tetraacetic acid |
| | PAGE | Polyacrylamide gel electrophoresis |
| | PBS | Phosphate buffered saline |
| | PCR | Polymerase chain reaction |
| | PFU | Plaque forming unit |
| 10 | PMSF | Phenylmethylsulphonyl fluoride |
| | poly(A) | Polyadenylic acid |
| | PV | Pasteur virus |
| | SDS | Sodium dodecyl sulphate |
| | Tris | Tris(hydroxymethyl) aminomethane |
| 15 | R | Ribozyme domain of TB-1 DNA construct |
| | R1 | Ribozyme 1 domain of TB-2 DNA construct |
| | R2 | Ribozyme 2 domain of TB-2 DNA construct |
| | RNA | Ribonucleic acid |
| | RNP | Ribonucleoprotein |
| 20 | rpm | Revolutions per minute |
| | RT | Room temperature |
| | VSV | Vesicular stomatitis virus |
| | VLP | Virus-like particle |
| | w/w | Percentage by weight |

25 MATERIALS AND GENERAL EXPERIMENTAL PROCEDURES

Synthetic oligonucleotides (see Figure 9) were supplied as deprotected and purified products by the Centre for Molecular Biology and Biotechnology, St Lucia, Brisbane, Australia.

30 All recombinant DNA cloning and analysis procedures including restriction enzyme digestions, dephosphorylations, ligations, transformations, DNA preparations and DNA electrophoresis were as described by Sambrook et al (1989) Molecular Cloning: A Laboratory
 35 Manual, 2nd Edition, Cold Spring Harbour Laboratory Press: New York and Perbal (1988) A practical Guide to Molecular Cloning, Wiley: New York.

Procedures for nucleotide sequence analysis were described in Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467.

5 Procedures for analysis of proteins including SDS-PAGE and immunoblotting were conducted as described in Prehaud et al. 1989 Virology 173:390-399; 1990, Virology 178:486-497.

10 Procedures for use of the baculovirus expression system including culture of *Spodoptera frugiperda* cells, construction of shuttle and transfer vectors and generation, selection and analysis of recombinant baculoviruses were conducted as described in Prehaud et al., 1989, Virology 173:390-399; 1990, Virology 178:486-497.

15

EXAMPLES OF THE PREFERRED MODE

Example 1 Construction of a plasmid vector containing the TB-2 DNA molecule

20 The TB-2 DNA is constructed by several consecutive PCRs by using overlapping synthetic oligonucleotide primers and a rabies genomic RNA template in the following steps:

(i) A plasmid vector containing the filler domain was obtained by using the rabies virus (CVS strain) genome, primer L1 (Figure 9) and reverse transcriptase to prepare a single-stranded cDNA copy of the required portion of the rabies L protein gene and then by using primers L1 and L2 (Figure 9) and the polymerase chain reaction (PCR) to amplify a double-stranded DNA molecule of the required nucleotide sequence. The DNA molecule was then cloned into the Sma I site of a suitable plasmid vector (eg. pUC118). The recombinant plasmid containing the filler domain was named pFILL. The complete nucleotide sequence of the recombinant DNA insert in pFILL was determined and shown to correspond to that of the filler

25

30

35

domain illustrated in Figure 5.

(ii) The filler domain was extended by PCR using primers TB5A and TB3A (Figure 9) and pFILL obtained in step (i) as template.

5 (iii) The PCR product obtained in step (ii) was extended by PCR using primers TB5B and TB3B (Figure 9).

(iv) The PCR product obtained in step (iii) was extended by PCR using primers TB5C and TB3C (Figure 9).

10 (v) The PCR product obtained in step (iv) was cloned after BAH H1 digestion in a suitable plasmid vector (eg pBluescript KS+, Stratagene, La Jolla, USA). The plasmid vector containing the TB-2 construct was named pTB2. The complete nucleotide sequence of the recombinant insert in pTB2 was determined and shown to correspond to that of the TB-2 construct illustrated in Figure 5.

20 Example 2 Construction of a plasmid vector containing the TB-1 DNA molecule.

The TB-1 DNA was derived from the TB-2 DNA construct by using PCR according to the following steps:

25 (i) The pTB-2 plasmid was used as a template for PCR using primers TB1 and TB3C (Figure 9).

(ii) The PCR product obtained in step (i) was extended by PCR using primers TB5C and TB3C (Figure 9).

30 (iii) The PCR product obtained in step (ii) was cloned after Bam H1 digestion in a suitable plasmid vector (eg pBluescript KS+, Stratagene, La Jolla, USA). The plasmid vector containing the TB-1 construct was named pTB1. The complete nucleotide sequence of the recombinant insert in pTB1 was determined and shown to correspond to that of the TB-1 DNA construct illustrated in Figure 8.

Example 3 *In vitro*transcription of TB-2 and TB-1
RNA and demonstration of ribozyme cleavage.

Plasmid vectors pTB2 and pTB1 were cut with Xba1 or uncut, and were used as templates for *in vitro* transcription by T3 RNA polymerase using the pGEM express transcription kit (promega, Rozelle, NSW, Australia) and [³⁵S]UTP (Amersham International Ltd). *In vitro* transcriptions were conducted at 37°C or at 28°C. The products were analysed by electrophoresis in 6% polyacrylamide-urea sequencing gels. A plasmid of known sequence was prepared in a standard dideoxynucleotide sequencing reaction and run in adjacent lanes as a molecular weight ladder. After electrophoresis, the gels were fixed and dried and visualised by autoradiography.

In vitro transcription at both 37°C or 27°C resulted in RNA transcripts that were of a size corresponding to the products of cis-acting cleavage at the sites targeted by the ribozyme domains. The results are illustrated in Figure 10 for transcription from plasmid pTB2 at 37°C. The short product of ribozyme R1 cleavage at the 5' end of the transcript appeared as a discrete band (A) of 93 nucleotides corresponding to the predicted sequence from the T3 transcription start to the R1 cleavage site. The short product of ribozyme R2 cleavage at the 3' end of the transcript appeared as a double band (B) of 66 and 67 nucleotides when using Xba 1-cut plasmid as template. The corresponded to the predicted sequence from the R2 cleavage site to the Xba 1 site and a 1 base run-on (known to often occur at the 3' end of *in vitro* transcripts). As expected, band B did not occur when uncut plasmid was used as template as the predicted product would be of large and variable size depending on the nature of transcription termination. The long cleavage product of R1 and R2 corresponding to the rabies sub-genomic RNA was located at band C. Similar results were obtained for TB-2 at 28°C although the transcription efficiency was lower. As expected, *in vitro*

transcription using Xba 1 cut pTB1 resulted only in band B and a large product of slightly smaller size to band C above.

5 The results indicated that both the 5' and 3' ribozyme domains were active and efficiently cleaved the transcripts derived from the DNA constructs to generate the desired sub-genomic RNA fragments. That cleavage occurred at 28°C indicated that the ribozyme domains were active at the ambient temperature of insect cells.

10 Example 4 Construction of recombinant baculoviruses containing the TB-2 and TB-1 DNA molecules.

Baculovirus (AcNPV) transfer vectors were constructed by obtaining the TB-2 and TB-1 DNA inserts from recombinant plasmids pTB2 and pTB1 respectively by 15 digestion with Bam H1, and subcloning into a Bam H1 digested, dephosphorylated derivative of the transfer vector pAcYM1 (NERC IVEM, Oxford, UK). The transfer vectors containing the TB-2 and TB-1 DNA inserts were named pActB2 and pActB1 respectively.

20 To obtain recombinant baculoviruses expressing the TB-2 and TB-1 subgenomic RNAs, *Spodoptera frugiperda* cells were lipofected with a mixture of recombinant transfer vector (pActB2 or pActB1) DNA (1 µg) and baculovirus ACRP23-lacZ viral DNA (100 ng). The 25 recombinant baculoviruses were selected as described previously (Kitts et al. 1990, Nucleic Acid Research 18:5667-5672; and Prehaud et al. 1992, Virology, in Press) and high titers stocks were prepared (i.e. $>10^7$ PFU/ml). The recombinant baculoviruses containing the 30 TB-2 and TB-1 DNA constructs were names Ac.TB2 and Ac.TB1 respectively.

Example 5 Mixed infections of *Spodoptera frugiperda* cells and purification of rabies VLPs.

35 Four identical cultures of *Spodoptera frugiperda* cells (5×10^7 cells) were co-infected at a multiplicity of 1PFU/cell with:

- (i) wild type baculovirus (AcNPV) and recombinant

- baculoviruses expressing the rabies sub-genomic RNA (Ac.TB2 or Ac.TB1); or
- (ii) recombinant baculoviruses expressing the rabies N protein (AcNPV3), M1 protein (AcNPVM1), M2 protein (AcNPVM2) and G protein (AcNPV2) (see Prehaud et al., 1989, Virology 173: 390-399; 1990, Virology 178: 486-497); or
- (iii) recombinant dual expression baculoviruses expressing the rabies N/M1 proteins (AcNPVN/M1) and M2/G proteins (AcNPVM2/G); or
- (iv) recombinant dual expression baculoviruses AcNPVN/M1 and AcNPVM2/G and recombinant baculoviruses expressing the rabies sub-genomic RNA (Ac.TB2 or Ac.TB1).

The cultures were incubated for 3 days at 38D. The culture supernatants were recovered from the cultures and clarified by a centrifugation at 4,000 rpm for 10 min at 4 in a JA20 rotor (Beckman). Particles were pelleted from the supernatant in an SW28 rotor (Beckman) for 1h at 27,000 rpm at 4°C. The pellet were resuspended in TD buffer (0.8 mM TrisOHCl, 150mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 10 mM EDTA, pH 7.4) and further purified by centrifugation in a two step sucrose gradient 10 to 40% (w/w) made in TD buffer. The band located at the interface of the gradient was collected and particles were pelleted by centrifugation in an SW40 rotor (Beckman) for 1hr at 30,000 rpm at 4°C. Pellets were resuspended in TD buffer and stored at -20°C. As illustrated in Example 7 and Example 8 below, this procedure resulted in the purification of VLPs only from cultures in which both rabies virus proteins and the TB02 or TB-1 subgenomic RNAs were expressed.

Example 6 Preparation of cell lysates from
mixedly-infected *Spodoptera frugiperda* cells.

Four identical cultures of *Spodoptera frugiperda* cells (1.5×10^6 cells) were co-infected at a multiplicity of 1 PFU/cell with wild type and recombinant

5 baculoviruses as described in Example 5 above. At 3 days after infection, the supernatant media were removed from the dishes, the monolayers rinsed three times with phosphate-buffered saline (PBS), and the cells lysed in 150 µl of RIPA buffer (1% Triton X-100, 1% sodium dodecyl sulphate, pH 7.4). Aliquots of the protein samples were boiled for 10 min in Dissociation buffer (2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, 0.01% bromophenol blue, pH6.8) and stored at -20°C.

10 Example 7 Analysis of proteins synthesised in mixedly infected *Spodoptera frugiperda* cells and in purified rabies VLPs.

15 Cell lysate preparations obtained as described in Example 6 above and gradient-purified particle preparations from the culture supernatant described in Example 5 above were analysed for the presence of rabies virus proteins by SDS-PAGE and immunoblotting. Proteins resolved by electrophoresis in a 10% SDS-polyacrylamide gel were electroblotted to a nitrocellulose membrane. 20 Blots were incubated for 2 h with the blocking solution (3% low fat skim milk powder and 0.01% sodium azide in PBS), then transferred to blocking solution containing a 1/500 dilution of a mouse anti-CVS polyclonal antibody and incubated overnight. After washing, the bound 25 antibody was detected using a peroxidase-conjugated anti-mouse IgG (Sigma Chemicals, St. Louis, MO, USA). The blots were developed by using 4-chloro-1-naphthol and 3-3'-diaminobenzidine tetrahydrochloride (Sigma Chemicals, St Louis, MO, USA) as substrate for the peroxidase.

30 Analysis of cell lysate preparations by this procedure resulted in detection of rabies virus G, N, M1 and M2 proteins in the lysates of cultures infected with single or dual recombinant baculovirus expression vectors containing the corresponding genes and in the lysate from 35 the cultures infected with the dual recombinant baculovirus AcNPVN/M1 and AcNPVM2/G and recombinant baculoviruses ACTB2 or ACTB1. No rabies virus proteins

were detected in cultures infected with wild type baculovirus and recombinant baculoviruses Ac.TB2 or Ac.TB1.

5 Analysis of gradient-purified particle preparations by this procedure resulted in detection of rabies virus N, M1, M2 and G proteins only in particles prepared from cultures infected with the dual recombinant baculovirus AcNPVN/M1 and AcNPVM2/G and recombinant baculoviruses AcTB2 or AcTB1. No reaction with rabies
10 antibody was detected in particle preparations from other cultures described in Example 5 above.

The results of these analyses are summarised in Figure 11. The results indicate that rabies N, M1, M2 and G proteins were synthesised in all cultures infected
15 with baculovirus expression vectors containing the corresponding genes. However, only in cultures which were also infected with recombinant baculoviruses expressing the TB-2 and TB-1 sub-genomic RNAs resulted in the release of particles containing the rabies proteins.
20 These particles were also identified by electron microscopy as described in Example 8 below.

Example 8 Electron microscopy of rabies VLP preparations

Purified fractions obtained in Example 5 above
25 from cultures mixedly-infected with wild type and recombinant baculoviruses were dropped onto carbon coated grids, washed three time with TD buffer and negatively stained with 2% uranyl acetate. The samples were then examined using an Hitachi transmission electron
30 microscope.

In control preparations from cultures co-infected with:

- (i) wild type baculovirus and recombinant baculoviruses expressing the rabies sub-genomic RNA; or
35
(ii) recombinant baculoviruses expressing the rabies N, M1, M2 and G proteins; or

(iii) recombinant dual expression baculoviruses expressing the rabies N/M1 proteins and M2/G proteins;

5 only rod shaped particles corresponding to known structures of baculovirus particles were observed. The particles were of a relatively consistent size (250 nm x 40 nm) and shape (rod shaped) and appeared to be largely intact.

10 In preparations from cultures co-infected with recombinant baculoviruses expressing the rabies N, M1, M2 and G proteins and recombinant baculoviruses expressing the rabies sub-genomic RNA two types of particle were observed (illustrated in Figure 12). One particle corresponded to the baculovirus particles observed in
15 control preparations. The other particle type (which was never observed in control preparations) comprises irregular structures of approximately 70-85 nm diameter. These particles often displayed an electron-dense core and a brightly illuminated perimeter through which
20 numerous regular surface spikes or projections protruded. These particles are the rabies VLPs and are similar to those previously described for some types of rhabdovirus DI particles (see *The Rhabdoviruses* (1987) Ed. RR Wagner Plenum Press, New York) in which the electron-dense core
25 represents the ribonucleoprotein complex, the brightly illuminated perimeter represents the viral lipid envelope and the spikes represent the viral surface glycoprotein.

SCOPE AND DEPOSITIONS ASSOCIATES WITH THE INVENTION

30 The invention also includes within its scope the aforementioned DNA constructs per se as well as the VLPs which may also be used for purposes other than a vaccine component ie. diagnostic reagent.

35 The plasmid pACTB2 was deposited at the Australian Government Analytical Laboratories (AGAL) Suarokin St. Pymble, N.S.W., Australia on July 16 1992 and was allocated Accession Number 92/32589.

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DESCRIPTION OF FIGURES

FIGURE 1: Schematic illustration of some DNA constructs that would be suitable for expression of a sub-genomic RNA of a rhabdovirus or paramyxovirus for inclusion in VLPs. R1 and R2 are suitable ribozyme domains; the 5' domain is derived from the 5' terminal sequence of a rhabdovirus or paramyxovirus (-) sense RNA genome; the 3' domain is derived from the 3' terminal sequence of a rhabdovirus or paramyxovirus (-) sense RNA genome; the filler domain is any suitable sequence of nucleotides; F1 and F2 are parts of the filler domain; and S1 and S2 are intervening nucleotide sequences.

FIGURE 2: Schematic illustration of the general process of rhabdovirus or paramyxovirus VLP formation using for example the formation of rabies VLPs using the baculovirus expression system.

FIGURE 3: Schematic illustration of the organisation of the TB-2 sub-genomic DNA construct. The structure is represented as a double-stranded DNA molecule which is suitable for cloning into the Bam HI site of a baculovirus expression vector. The R1 and R2 ribozyme cleavage sites are those which are active in the RNA transcript of this cloned DNA assuming transcription occurs in the direction indicated.

FIGURE 4: Illustration of the sequence of the transcript of the TB-2 sub-genomic DNA construct indicating the functional domains and the R1 and R2 ribozyme cleavage sites.

FIGURE 5: Nucleotide sequence of the TB-2 sub-genomic DNA construct (single-stranded DNA in the transcription [+] sense).

FIGURE 6: Schematic illustration of the organisation of the TB-1 sub-genomic DNA construct. The structure is represented as a double-stranded DNA molecule which is suitable for cloning into the Bam HI site of a baculovirus expression vector. The R ribozyme cleavage site are those which are active in the RNA

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transcript of this cloned DNA assuming transcription occurs in the direction indicated.

FIGURE 7: Illustration of the sequence of the transcript of the TB-1 sub-genomic DNA construct indicating the functional domains and the R ribozyme cleavage site.

FIGURE 8: Nucleotide sequence of the TB-1 sub-genomic DNA construct (illustrated as a single-stranded DNA in the transcription [+] sense).

FIGURE 9: Nucleotide sequence of synthetic oligonucleotides used for PCR in the construction of the filler domain, the TB-2 sub-genomic DNA and the derived TB-1 sub-genomic DNA.

FIGURE 10: Autoradiograph of a 6% polyacrylamide-urea gel indicating the products of *in vitro* transcription of uncut and Xba I-cut pTB2 DNA at 37°C using T3 RNA polymerase. Ribozyme cleavage products are identified as bands A, B and C as described in the text above.

FIGURE 11: Immunoblot of cell lysates and particle preparations from mixed infections with recombinant baculoviruses using polyclonal anti-rabies mouse serum as described in the text above. Lane 1: Lysate of cells infected with wild type AcNPV; Lane 2: Lysate of cells infected with recombinant dual expression baculoviruses expressing rabies N/M1 proteins and M2/G proteins; Lane 3: Lysate of cells infected with recombinant single expression baculoviruses expressing rabies N, M1, M2 and G proteins; Lane 4: Particles prepared from the culture supernatant of cells infected with recombinant dual expression baculoviruses expressing rabies N/M1 proteins and M2/G proteins and recombinant baculovirus ActB2 containing the TB-2 DNA construct; Lane 5: Particles prepared from the culture supernatant of cells infected with recombinant dual expression baculoviruses expressing rabies N/M1 proteins and M2/G proteins.

FIGURE 12: Electron micrograph of rabies VLP

preparation. The sample was prepared as described in the text above from the culture supernatant obtained from a mixed infection of *Spodoptera frugiperda* cells with recombinant baculovirus vectors expressing the rabies N, M1, M2 and G proteins and a recombinant baculovirus expression vector Ac.TB2. As described in the text above, the micrograph illustrates both rabies VLPs (irregular particles 70-85 nm diameter, with surface projections) and particles of the recombinant baculoviruses (rod-shaped particles 40 x 250 nm).

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CLAIMS:

1. A process for producing a vaccine for treatment of infections caused by rhabdoviruses and paramyxoviruses including the steps of:

5 (i) constructing a DNA molecule corresponding to a complete genome, modified genome or genome fragment of a rhabdovirus or paramyxovirus comprising a 3' domain, a filler domain and a ribozyme domain; and

10 (ii) inserting the DNA obtained in step (i) into the cloning site of a eukaryote expression vector and transfecting a eukaryote cell with the vector containing the genome construct and simultaneously transfecting the same eukaryote cell with vectors containing cloned genes of rhabdovirus or paramyxovirus structural proteins including those with similar functions to the G protein, N protein, M₁ protein and M₂ protein of rabies virus; and

15 (iii) obtaining from the cell transfected in step (ii) virus like particles (VLPs) consisting of an RNA genome transcribed from the DNA molecule constructed in step (i) surrounded by a sheath of N protein and M₁ protein to form a ribonucleoprotein complex and a lipid envelope including the G protein and an internal matrix comprising the M₂ protein; and
20 (iv) including the VLPs obtained in step (iii) in a vaccine .

25 2. A process as claimed in claim 1, wherein in step (i) the DNA molecule additionally comprises a 5' domain and additional ribozyme domain to ensure that the expressed RNA is cleaved at the 5' terminus.

30 3. A process as claimed in claim 1, wherein in step (ii) there is additionally included a cloned gene corresponding to the rabies L protein or similar protein in other rhabdovirus or paramyxovirus.
35

4. A process as claimed in claim 1, wherein the DNA molecule in step (i) has cohesive ends.

5. A process as claimed in claim 1, wherein the DNA molecule in step (iii) includes a filler domain comprising L protein from rabies virus or similar protein from other rhabdovirus or paramyxovirus.

6. A process as claimed in claim 1, wherein in step (ii) the eukaryote expression vector is baculovirus and the eukaryote cell is Spodoptera frugiperda.

7. A process as claimed in claim 1, wherein in step (iii) the VLPs are combined with an adjuvant.

8. A vaccine prepared from the process of claim 1.

9. A virus-like particle (VLP) containing an RNA genome including a 3' domain and a filler domain surrounded by a sheath of rabies N protein or similar protein from a rhabdovirus or paramyxovirus and rabies M₁ protein or similar protein from a rhabdovirus or paramyxovirus to form a ribonucleoprotein complex and a lipid envelope of rabies G protein or similar protein from a rhabdovirus or paramyxovirus and an internal matrix of rabies M₂ protein or similar protein from a rhabdovirus or paramyxovirus.

10. A vaccine for rhabdovirus or paramyxovirus infection including the VLP of claim 9.

11. A vaccine as claimed in claim 10 including an adjuvant.

12. A DNA construct including a 3' domain, a ribozyme domain to ensure that any RNA expressed from the DNA construct is expressed at the 3' terminus and a filler domain.

13. A DNA construct as claimed in claim 14 further including a 5' domain and a further ribozyme domain to ensure that any RNA expressed from the DNA construct is expressed at the 5' terminus.

14. DNA constructs described in Figure 1 herein.

15. TB1 herein.

16. TB2 herein.

- 17. DNA construct Ac - TB2.
- 18. Plasmid pACTB2.

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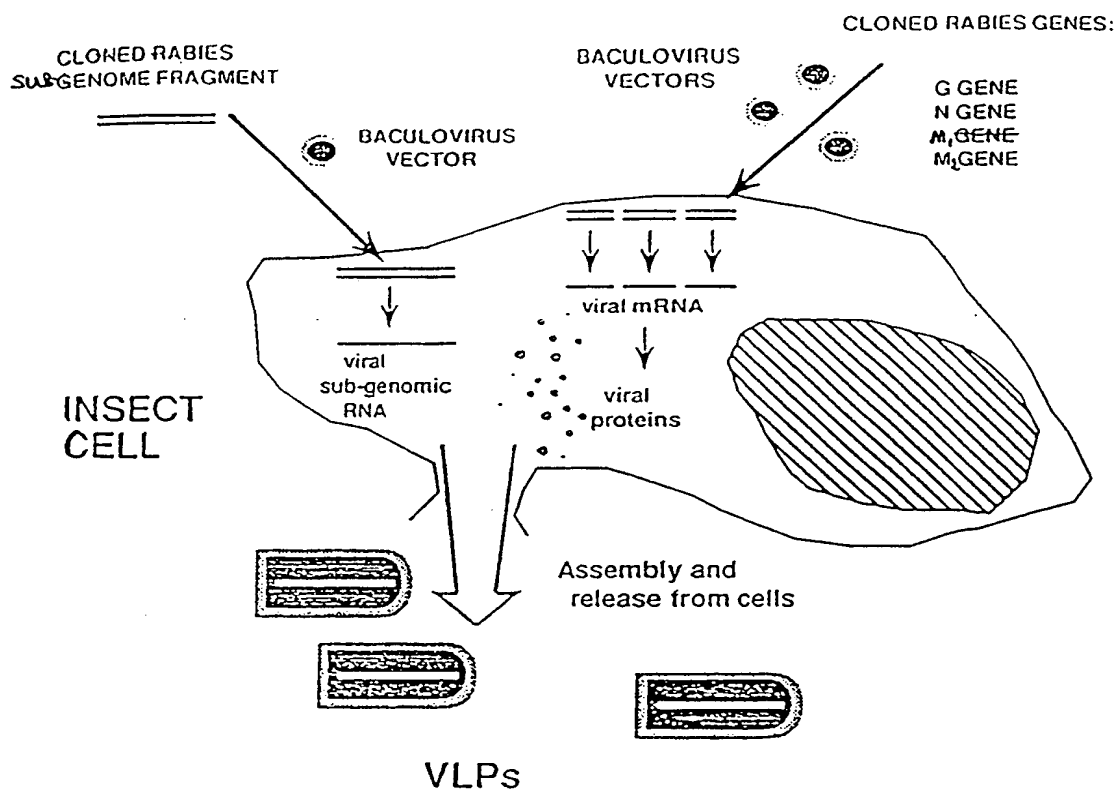
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FIGURE 1

- (A) R1 - S1 - 5'domain - filler domain - 3' domain - S2 - R2
- (B) 5'domain - R1 - filler domain - R2 - 3' domain
- (C) R1 - S1 - 5'domain - filler domain - R2 - 3' domain
- (D) 5'domain - R1 - filler domain - 3' domain - S2 - R2
- (E) R1 - 5'domain - filler domain - 3' domain - R2
- (F) 5'domain - F1 - R1 - filler domain - R2 - F2 - 3' domain
- (G) S1 - filler domain - 3' domain - R2
- (H) S1 - filler domain - R2 - 3' domain
- (I) 5'domain - F1 - R1 - filler domain - 3' domain - S2 - R2
- (J) R1 - S1 - 5'domain - filler domain - R2 - F2 - 3' domain

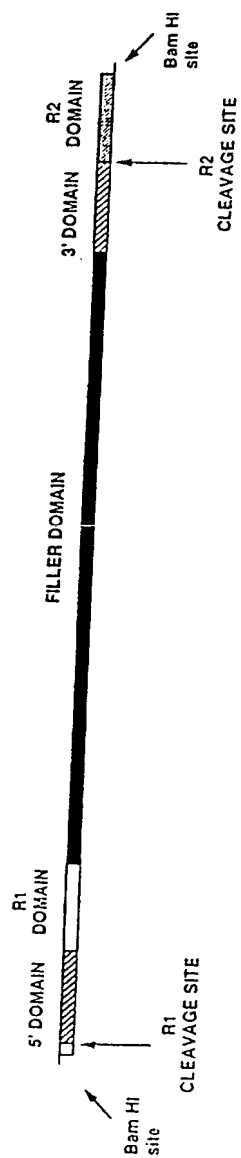
2/12

Figure 2: Construction of rabies VLPs



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FIGURE 3: TB-2 SUB-GENOMIC DNA CONSTRUCT ORGANIZATION



FUNCTIONAL STRUCTURE OF THE RNA TRANSCRIPT OF TB-2

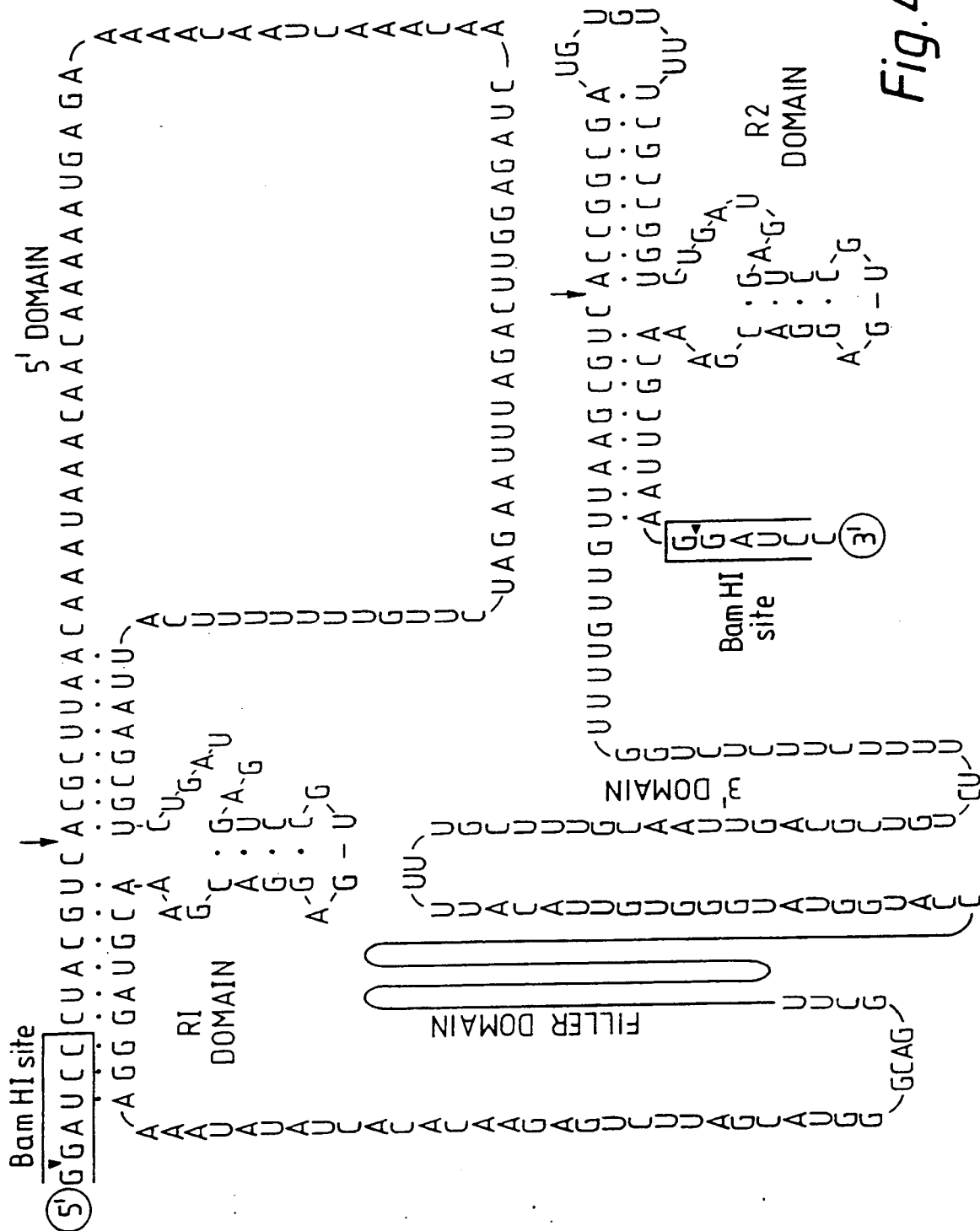


Fig.4.

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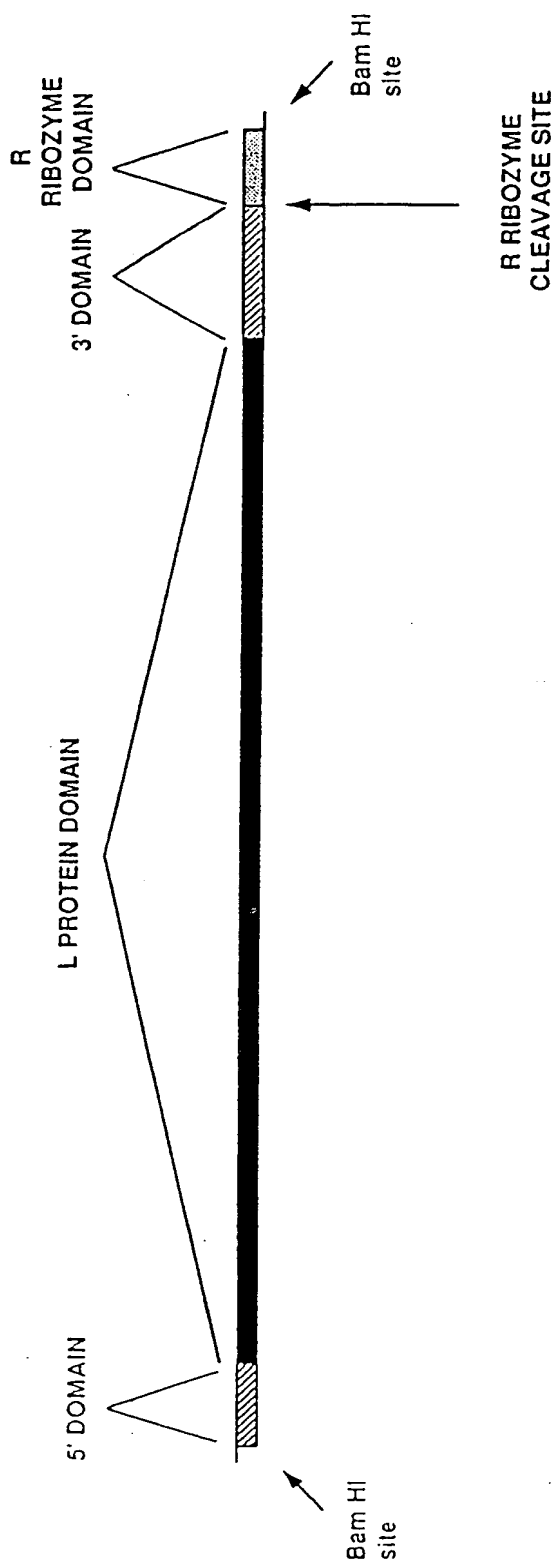
FIGURE 5: SEQUENCE DESCRIPTION TB-2

| | |
|---|------|
| GGATCCCTAC GTCACGCTTA ACAAATAAAC AACAAAAATG AGAAAAACAA TCAAACAAC | 60 |
| AGAGGTTTCAG ATTTAAGATC TTGTTTTTTT CATTAAAGCGT CTGATGAGTC CGTGAGGACG | 120 |
| AAACGTAGGG AAAATATATC ACACAAGAGT CTTAGCATGG GCAGGCTTTC GGTAATATCC | 180 |
| GGCTTACAAA CGACTGTAAT CTAGTAGGGA TGACTAGATC GGATATCCTC GAGGGTCATC | 240 |
| CATCTGTTAT ACCTATGAAG ATGTCGTTCT ACCTCTCCTG ATAGGTCTTT TATGCTGCCA | 300 |
| ATGAGTCTGG TAGTCTTCAC TATCTTGTAG ATCAACCTGA TCCAGTGAGA TGACAGACTC | 360 |
| AAGCTAGAAT TACAGGCTAC TCTCTTGAAC ACTGGGGTAT CATCGGACCA ACTCCAAGAT | 420 |
| AGATAAATAT TCCCTCGAAT AACTTGTTTT CTGAAGTAAC AAGTTATAGG TCTGTTATAC | 480 |
| AGGTCATGAA GTCTTGCGAA GTTAGGGACG TCGCCTAAAG CGGTAGATAG ATACATCATA | 540 |
| GTACTGCAAC ATATGTTGAA GTGCCTCAGG ATTTTAGGAT CAGATTCAGG GGGTAGAACA | 600 |
| AGGGGGTCAG TCAAAGGTTT GGAAATGTTG AAGACTCTAT TGGAAAAAAC GATCATGATG | 660 |
| GATATAATGA TAGCCACTTT AGACAGAGTT CCCCTCTGTA GCTCAAGATC ATCCACCATC | 720 |
| TTGTGGACTA GGAAGGACTC TACATCATTG TCAATCAGAG TTATGATCAT CTCGTTGTAA | 780 |
| GGATTTGATA TGATCTCTTC AGGAAATCCC CTTACCAGGT CTTGATAGTT TAAGGAACGA | 840 |
| GCTCTCTGCA TCTCACTTTT GGGGCTGCTG CAATTGAACA ACACAAGGCT CATCTCTCGA | 900 |
| AGGGTGGAAG AGGTCAAGTA CCGGCGTCCC TGAAAACTT TCCTCGTTTA GAGAACCGGA | 960 |
| GTATAGCTCA GAAGAAAAGG ATGAAGTTAC TTGGGTTACA AACCCTGTGA CCGAAGGAAC | 1020 |
| GCTCTCGACA GATGTTGAAT AGCTTTATAG TCCGGGTTTA CTAGCATAGT CCCGTAAGTT | 1080 |
| TTGAAGACCA GATAAAGTGG TCCATCTATA GACAATGCGA AATCAGACAT CAACAGAGTT | 1140 |
| ATCCGGTTGA TAGATGCAAT ATCAGTAACT TCTGCATCAC AAATAATGAG GTCATACGAC | 1200 |
| ATGTTGACCT GTTTTTGAAC CGACTGGAAG TATCTCCAGG TGGCCGAGTT CCTCAGGTCG | 1260 |
| GACGGTTTCT CCCAGATTGA GTCAAAATCT ATCACTCTCC ATGGTATGGG TGTTACATTT | 1320 |
| TTGCTTTGCA ATTGACGCTG TCTTTTTCTT CTCTGGTTTT GTTGTTAAGC GTCACCGGCG | 1380 |
| ATGTGTTTTT GCCGGTCTGA TGAGTCCGTG AGGACGAAAC GCTTAAGGAT CC | 1432 |

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FIGURE 6: TB-1 SUB-GENOMIC DNA CONSTRUCT ORGANIZATION



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FIGURE 8: SEQUENCE DESCRIPTION TB-1

| | |
|--|------|
| GGATCCCTAC GTCACGCTTA ACAAATAAAC AACAAAAATG AGAAAAACAA TCAAACAAC | 60 |
| AGAGGTTTACG ATTTAAGATC TTGTTTTTTT CAAAATACAT CACACAAGAG TCTTAGCATG | 120 |
| GGCAGGCTTT CGGTAATATC CGGCTTACAA ACGACTGTAA TCTAGTAGGG ATGACTAGAT | 180 |
| CGGATATCCT CGAGGGTCAT CCATCTGTGA TACCTATGAA GATGTCGTTT TACCTCTCCT | 240 |
| GATAGGTCTT TTATGCTGCC AATGAGTCTG GTAGTCTTCA CTATCTTGTA GATCAACCTG | 300 |
| ATCCAGTGAG ATGACAGACT CAAGCTAGAA TTACAGGCTA CTCTCTTGAA CACTGGGGTA | 360 |
| TCATCGGACC AACTCCAAGA TAGATAAATA TTCCCTCGAA TAACTTGTTT TCTGAAGTAA | 420 |
| CAAGTTATAG GTCTGTTATA CAGGTCATGA AGTCTTGCGA AGTTAGGGAC GTCGCCTAAA | 480 |
| GCGGTAGATA GATACATCAT AGTACTGCAA CATATGTTGA AGTGCCTCAG GATTTTAGGA | 540 |
| TCAGATTGAG GGGGTAGAAC AAGGGGGTCA GTCAAAGGTT TGGAAATGTT GAAGACTCTA | 600 |
| TTGGAAAAAA CGATCATGAT GGATATAATG ATAGCCACTT TAGACAGAGT TCCCCTCTGT | 660 |
| AGCTCAAGAT CATCCACCAT CTTGTGGACT AGGAAGGACT CTACATCATT GTCAATCAGA | 720 |
| GTTATGATCA TCTCGTTGTA AGGATTTGAT ATGATCTCTT CAGGAAATCC CCTTACCAGG | 780 |
| TCTTGATAGT TTAAGGAACG AGCTCTCTGC ATCTCACTTT TGGGGCTGCT GCAATTGAAC | 840 |
| AACACAAGGC TCATCTCTCG AAGGGTGGAA GAGGTCAAGT ACCGGCGTCC CTGAAAAACT | 900 |
| TTCCTCGTTT AGAGAACCGG AGTATAGCTC AGAAGAAAAG GATGAAGTTA CTTGGGTTAC | 960 |
| AAACCCTGTG ACCGAAGGAA CGCTCTCGAC AGATGTTGAA TAGCTTTATA GTCCGGGTTT | 1020 |
| ACTAGCATAG TCCCGTAAGT TTTGAAGACC AGATAAAGTG GTCCATCTAT AGACAATGCG | 1080 |
| AAATCAGACA TCAACAGAGT TATCCGGTTG ATAGATGCAA TATCAGTAAC TTCTGCATCA | 1140 |
| CAAATAATGA GGTCATACGA CATGTTGACC TGTTTTTGAA CCGACTGGAA GTATCTCCAG | 1200 |
| GTGGCCGAGT TCCTCAGGTC GGACGGTTTC TCCCAGATTG AGTCAAAATC TATCACTCTC | 1260 |
| CATGGTATGG GTGTTACATT TTTGCTTTGC AATTGACGCT GTCTTTTCT TCTCTGGTTT | 1320 |
| TGTTGTTAAG CGTCACCGCG ATGTGTTTTT GCCGGTCTGA TGAGTCCGTG AGGACGAAA | 1380 |
| CGCTTAAGGA TCC | 1393 |

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FIGURE 9 Nucleotide sequence of oligonucleotides used.**TB5A:**

5' TGATGAGTCC GTGAGGACGA AACGTAGGGA AAATACATCA CACAAGAGTC TTAGCATGGG 60
CAGGCTTCCA GGAGTA 3' 76

TB5B:

5' ATCAAACAAC TAGAGGTTCA GATTTAAGAT CTTGTTTTTT TCATTAAGCG TCTGATGAGT 60
CCGTGAGGAC GAAACG 3' 76

TB5C:

5' GGCCGGATCC CTACGTCACG CTTAACAAT AAACAACAAA AATGAGAAAA ACAATCAAAC 60
AACTAGAGGT TCAGATT 3' 77

TB3A:

5' AGACAGCGTC AATTGCAAAG CAAAAATGTA ACACCCCTAC CATGGAGAGT GATAGATTTT 60
GACTCAATC 3' 69

TB3B:

5' AAACACATCG CCGGTGACGC TTAACAACAA AACCAGAGAA GAAAAAGACA GCGTCAATTG 60
CAAAGCAAA 3' 69

TB3C:

5' GGCCGGATCC TTAAGCGTTT CGTCCTCAG GACTCATCAG ACCGGCGAAA ACACATCGCC 60
GGTGACGCTT AA 3' 72

TB1:

5' ATCAAACAAC TAGAGGTTCA GATTTAAGAT CTTGTTTTTT TCAAATACA TCACACAAGA 60
GTC T 3' 64

L1:

5' AGAGTGATAG ATTTTGACTC AATC 3' 24

L2:

5' TCGGTAATAT CCGGCTTACA AACG 3' 24

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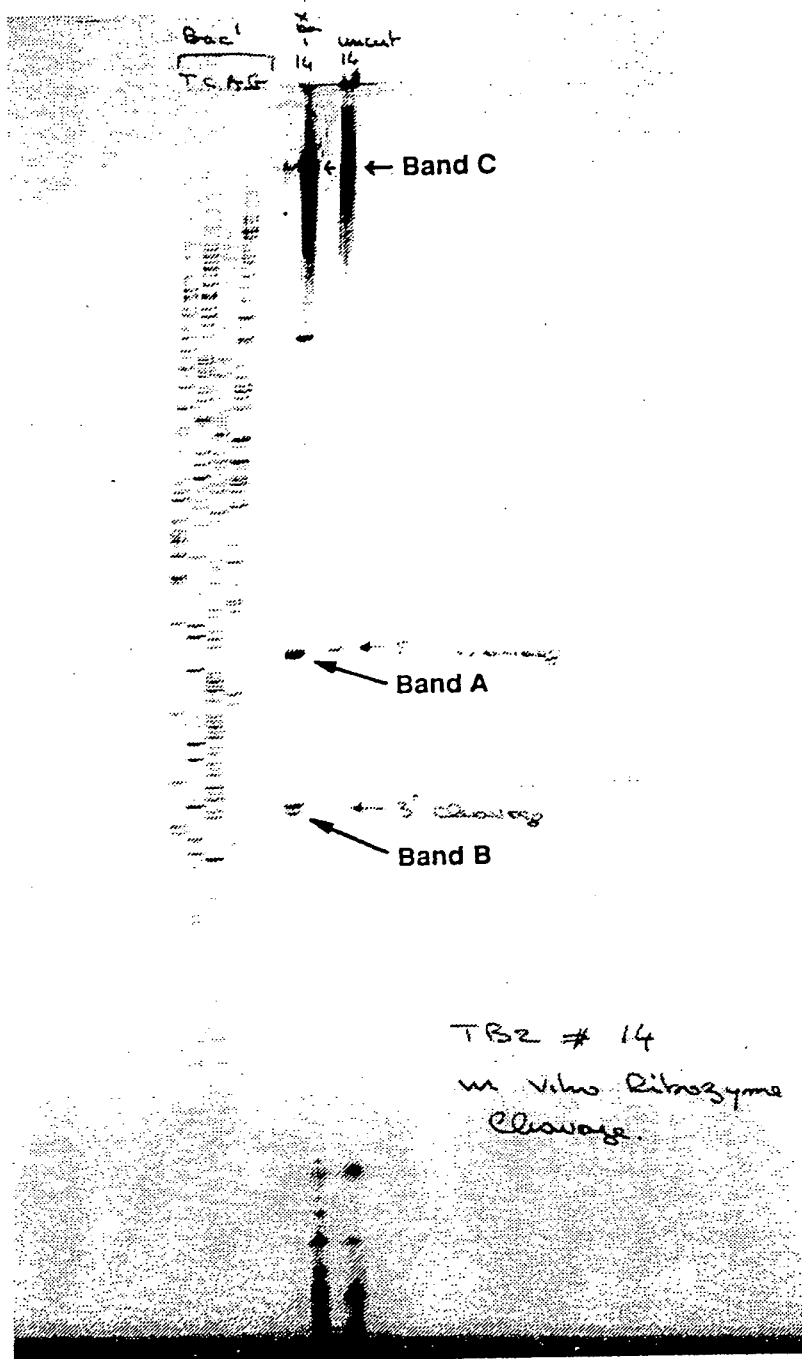


FIG 10

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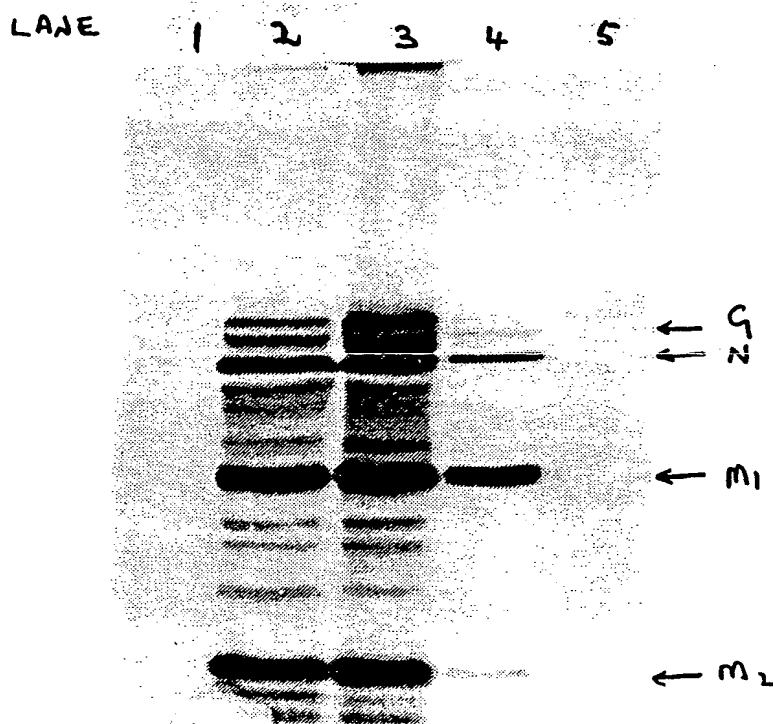


Fig. 11

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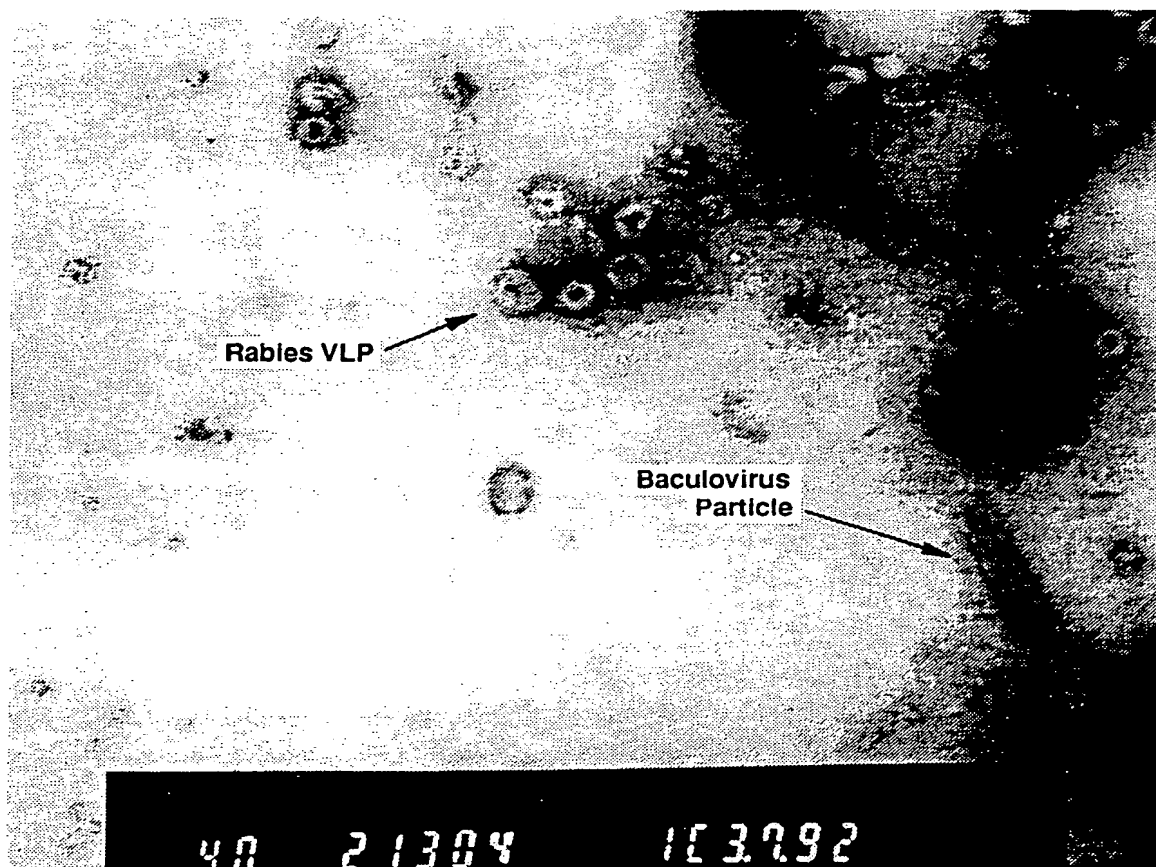


FIG 12

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl.⁵ A61K 39/175, A61K 39/205, C12N 7/01, C12N 15/47, C12N 15/45, C12N 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

AU:IPC as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

Chem Abs - Keywords - Rhabdovirus, Paramyxovirus, Rabies

Derwent - Rhabdovirus, Paramyxovirus, Rabies

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|-----------|--|-----------------------|
| A | Virology, Vol. 173(2) pages 390-399, issued 1989 (Oxford, UK) Prehaud et al "Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors" | 1-18 |
| A | Wistar Symp. Ser. (WSYSD3), Vol. 3 pages 259-267, issued 1985 (France) Lecocq et al, "New rabies vaccine: recombinant DNA approaches" | 1-18 |

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search
27 October 1992 (27.10.92)

Date of mailing of the international search report

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| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category* | Citation of document, with indication, where appropriate of the relevant passages | Relevant to Claim No. |
| A | Proc. Natl. Acad. Sci. USA (PNASA6), Vol. 81(22) pages 7194-7198, issued 1984, Wiktor et al, "Protection from rabies by vaccinia virus recombinant containing the rabies virus glycoprotein gene" | 1-18 |
| A | WO 87/02058 (THE UPJOHN CO.), 9 April 1987 (09.04.87) See whole document | 1-18 |
| A | EP 0256677 (E.I. DU PONT DE NEMOURS AND CO.), 24 February 1988 (24.02.88) See whole document | 1-18 |
| A | WO 90/02566 (MOLECULAR ENGINEERING ASSOCIATES INC.), 22 March 1990 (22.03.90) See whole document | 1-18 |

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | |
|--|--------|----------------------|----------|
| EP | 256677 | CA | 1301674 |
| | | JP | 63087976 |

